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13. ABSTRACT *(Maximum 200 Words)*

Excessive activation of growth factor receptors can lead to the unrestrained cellular proliferation characteristic of tumors. Our objective is to determine if SNX1, a protein involved in intracellular membrane trafficking, can be used to downregulate EGF receptors in mammary gland. Our approach is to characterize the gene for SNX1 and to generate transgenic animals overexpressing SNX1 in mammary glands. We have characterized a genomic clone for SNX1 and had planned to use this clone for transgenic vector construction. However, the size of the first intron in SNX1 wass too large for this approach to be used successfully. Instead, a WAP-SNX1 cDNA vector was constructed and used to generate transgenic animals. Out of appoximately 150 oocyte microinjections over a period of 6 months, only one transgene positive mouse was identified. This mouse (a female) was bred and delivered several litters but no transgenic offspring were detected

A Career Development Award was a second component of the application. Career development activities include: participation as reviewer on the American Cancer Society Cell Structure and Metastasis study section, participation on a search committee charged with identifying a Director for Breast Cancer Research at UAMS and participation as a reviewer on the California Breast Cancer Research Program Pathogenesis Study Section. In addition, the State of Arkansas Breast Cancer Research Program awarded me a one-year pilot research grant to examine the relationship between HER-2/*neu* and EGF receptors in mammary gland cell proliferation. In Fall 2001, the UAMS Promotion and Tenure committee approved my application for promotion to Associate Professor with tenure.

14. SUBJECT TERMS
breast cancer, down regulation, sorting nexin, transgenic, EGF receptor, Tumorigenesis, TGFα

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N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Richard C. Kurten April 13, 2002

PI - Signature Date

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INTRODUCTION

Inhibiting Tumorigenesis by Growth Factor Receptor Down Regulation Using a Sorting Nexin

The development of the mammary gland and its normal function in lactation is controlled by a variety of steroid hormones and peptide growth factors whose concentrations vary depending on the functional status of the gland. Disruption of this complex signaling network by genetic damage, environmental toxins or other factors can override normal restraints on cell growth and lead to the formation of tumors. Epidermal growth factor (EGF) levels been found to be elevated in breast tumor tissues and are an indicator of poor survivability because these tumors are generally resistant to therapies aimed at interruption of estrogen action. As a consequence of elevated EGF receptor levels, proliferative responses to growth factors are enhanced. This can result in hyperplasia and an increased probability of mutations occurring that further contribute to unrestrained tumor growth.

To prevent excessive mitogenic signaling, ligand bound receptors are removed from the cell surface in clathrin coated pits. Once inside the cell, receptor-ligand complexes are sorted away from nutritional receptors like the LDL receptor and targeted for degradation in lysosomes. The membrane trafficking events underlying lysosomal targeting involve the recognition of small amino acid "codes" by the sorting machinery. I recently discovered a protein, the sorting nexin, that recognizes the EGF receptor targeting code and stimulates its transport to the lysosome. In tissue culture cells, overexpression of sorting nexin 1 (SNX1) down regulated EGF receptors by dramatically shortening receptor half-life. As a consequence, EGF-stimulated receptor tyrosine kinase and immediate early gene activities were inhibited substantially. To test the hypothesis that SNX1 can be used *in vivo* to regulate mitogenic signaling by down regulating the EGF receptor, thereby inhibiting tumorigenesis, I propose to use an established transgenic mouse model for therapeutic intervention. In transgenic mice engineered to overexpress transforming growth factor alpha (TGFα), a ligand for the EGF receptor, mammary gland tumors invariably occur in females after 2 or 3 pregnancies. To determine if it is feasible to inhibit TGFα induced mammary gland tumorigenesis by overexpressing SNX1, the following technical objectives are proposed:

1. Isolate and characterize the human SNX1 gene using the SNX1 cDNA.
2. Prepare and characterize a transgenic mouse line with SNX1 expression targeted to the mammary gland using the whey acidic protein (WAP) promoter.
3. Cross SNX1 mice with TGFα mice and measure mammary gland tumor incidence to determine if SNX1 can be used as a tumor suppressor.

BODY

Technical Objective 1. Isolate and characterize the human SNX1 gene using the SNX1 cDNA that I cloned.

Task 1: **Months 1-2: Plate and screen genomic phage library by hybridization with ^{32}P labeled SNX1 cDNA. Prepare plaque pure phage stocks.**

Progress: In collaboration with H. Steven Wiley, University of Utah School of Medicine, 3 bacterial artificial chromosomes (BAC) containing the human SNX1 gene were isolated. Fluorescence *in situ* hybridization was used to determine the chromosomal localization of both SNX1 and SNX2 (Figure 1). Three different P1 clones were used to deduce the chromosomal localization of SNX1 and 2 were used for SNX2. Hybridization efficiency ranged from 85 to 91%. SNX1 was localized to human chromosome 15q22 and SNX2 was localized to chromosome 5q23.

Task 2: **Months 3-4: Purify phage DNAs and characterize the phage inserts by restriction mapping and Southern hybridization**

Progress: One of the BAC clones for SNX1, labeled 6K1, was characterized by subcloning and restriction mapping (Figure 2). Characterization of multiple clones was not practical due to the unexpectedly large size of the SNX1 gene.

Task 3: **Months 5-6: Subclone phage inserts that appear to contain the entire SNX1 coding region; confirm that they do by sequencing using primers complimentary to the ends of the SNX1 coding region and select the largest one for further use.**

Progress: The complete SNX1 coding region lies within 43kb of genomic DNA as defined by sequencing and oligonucleotide hybridization (Figure 2).

Task 4: **Month 8-12: Determine the complete sequence of the SNX1 gene.**

Progress: We have sequenced 15.7kb of the SNX1 gene. This represents 15 exons and considerable intronic sequence. Using this information and and an unordered working draft of sequences from human chormosome 15 submitted to GenBank by the Washington University genome Sequencing Center, we have generated a 99.2% sequenced 70.5kb map containing the human SNX1 structural gene between bp9860 and 53415. This completely defines the intron-exon structure of the SNX1 gene and the intron-exon boundaries (Figure 2).

Task 5: **Months 11-12: Prepare annual project report and a manuscript describing the human SNX1 gene.**

Progress: An annual report was prepared and submitted. A manuscript entitled "Structural and Functioanl Characterization of the Human Gene for Sorting Nexin 1 (SNX1) was published in **DNA and Cell Biology**. This work was also presented in poster format at the Department of Defense Breast Cancer Research Program "Era of Hope Meeting" in June 2000.

Technical Objective 2. Prepare a transgenic mouse line with SNX1 expression targeted to the mammary gland using the WAP promoter.

Task 6: **Months 7-9: Prepare the transgenic expression vectors.**

Progress: Transgenic expression vectors were prepared. The SNX1 genomic clone was too large for the proposed construction. Therefore we have adopted a cDNA approach that has been use successfully for stromolysin 1 (1) and for IGF (2). We obtained plasmid pbl103 containing bases -949 to +33 of the rat whey acidic protein (WAP) gene and plasmid pbTAPW3' containing 843 base pairs of WAP 3' sequence including the exons 3, 4 and the 3' untranslated region from Jeffrey Rosen, Baylor College of Medicine, Houston, Texas. We used these plasmids to generate a WAP-SNX1 cDNA vector. Our approach was to ligate the SNX1 cDNA into a WAP vector consisting of 943bp rat WAP 5' sequence and 675 bp WAP 3' sequence (Figure 3). To generate this construction, a 5' WAP PCR product was generated using oligonucleotides

5'-GATCGTCGACAAGGAGTATGGGCTGCACCA-3'

5'-GATCGAATTCGGCGGCGGCAGGCAAGTGAT-3'

as primers and pbl103 as the template. This PCR product contains rat WAP sequences -949 to -7. The 5' WAP PCR product was cleaved with SalI and EcoRI and cloned into the vector pBSIIKS(+) to generate pWAP 5'. Next, a 3' PCR product was generated using oligonucleotides

5'-GATCGAATTCAATGGCTGTATCATGAGTTG-3'

5'-GATCGCGGCCGCTCATTCTGTCAAGAGCTCAG-3'

as primers and pbTAPW3' as the template. The 3'WAP PCR product was cleaved with EcoRI and NotI and cloned into EcoRI/NotI cleaved pWAP 5' to generate pWAP 5' & 3' (Figure 3). The high fidelity polymerase Pfu was used for all PCR reactions reactions. An EcoRI fragment of SNX1 was cloned into the EcoRI site in pWAP 5' & 3'. The correct orientation of the SNX1 insert was determined by restriction enzyme mapping and nucleotide sequencing. The resultant plasmid, pWAP-SNX1cDNA was cleaved with SalI and NotI and the linearized minigene purified for mouse oocyte microinjection (Figure 4).

Task 7: **Months 10-13: Generate founder mice in collaboration with Jeffrey M. Rosen, Baylor College of Medicine.**

Progress: The pWAP-SNX1cDNA vector was constructed and a linearized DNA fragment was purified and sent to the NICHD transgenic Mouse Development Facility at the University of Alabama at Birmingham for the production of transgenic mice. A total of 22 mice were born in three litters during the period December 2000 and March 2001. Of the 22 mice, 6 died prior to weaning. In July 2001, we received 5 mice of which we confirmed one positive for the SNX1 transgene. This animal was breed and delivered one litter. The transgene was not detected in any of the pups. At this time, mouse hepatitis C was detected in our barrier facility and mouse breeding was suspended for several months.

Task 8: Months 16-18: Identify transgenic lines, confirm SNX1 protein expression.
Progress: A PCR assay was developed and validated for detecting the SNX1 transgene in FVB mouse tail blot DNA (figure 5). A graduate student, Anthony Eddington M.S., has been responsible for this aspect of the project. Of the 16 mice that survived to weaning, we have identified one transgene-positive female mouse in our PCR assay (figure 6). We bred this mouse and screened for transgene inheritance without success. For analysis of protein expression, we generated two new antibodies were generated that work well in western immunoblotting experiments (figure 7). Laboratory stocks of an affinity-purified polyclonal anti-peptide antibody (#3904) that was to previously used to measure SNX1 protein expression were depleted.

Task 9: **Months 19-22: Determine the effect of overexpressing SNX1 in the mammary gland on the concentration of mammary gland EGF receptors**

Progress: We have initiated experiments to measure EGF receptor levels in saturation binding experiments. We have encountered batch to batch variability in the quality of commercial ^{125}I-EGF preparation and are now iodinating our own EGF. In a separate project, we have analyzed several human breast tumor cell lines and use this to validate the ligand binding assay (figure 8). A technician, Ms. Susan Foreman, has been identified in a colleague's laboratory adjacent to my own to perform the assays. Susan has experience in more difficult measurements of β_2-adrenergic receptors in crude membranes and should have no problems performing the more sensitive EGF binding assays to measure EGF receptors in mouse mammary glands.

Task 10: **Months 23-24: Prepare annual project report and a manuscript describing the WAP-SNX1 mice and the consequences of SNX1 overexpression on EGF receptor levels in vivo.**

Progress: This annual report was prepared. Preparation of a manuscript has not been initiated as that awaits the completion of tasks 8-9.

Technical Objective 3. Cross SNX1 mice with TGFα mice and measure mammary gland tumor incidence to determine if SNX1 can be used as a tumor suppressor.

Progress: The tasks in technical objective (task 11-14) cannot be begun until task 8 is completed. A 12 month no-cost extension of the expiration date of the grant was requested and granted. However, despite success with technical procedures (vector construction, oocyte microinjection, transgene screening) we were unable to generate a transgenic mouse line.

KEY RESEARCH ACCOMPLISHMENTS

- Chromosomal Mapping of SNX1 and SNX2
- Characterization of the 60kb human SNX1 gene
- Analysis of alternative splicing of SNX1 in cDNA from human tissues
- Functional identification of the SNX1 promoter
- Completion of WAP-SNX1 minigene construction

- Validation of PCR assay to detect SNX1 minigenes in mouse tail DNA
- Generation and characterization of affinity-purified antibodies to detect the SNX1 protein
- 3 rounds of WAP-SNX1 minigene microinjeciton into oocyte yielding 22 mice
- Identification of 1 WAP-SNX1 minigene positive mouse

REPORTABLE OUTCOMES

IDEA Award: Work completed for Technical Objective 1 was presented in poster format at the department of defense Breast Cancer Research Progam "Era of Hope Meeting" in June 2000. A manuscript was published in DNA and Cell Biology.

Career Development Award: Work from my laboratory was presented in April 2000 at the American Association of Cancer Research meeting (Appendix 2) and at the California Breast Cancer Symposium in March 2002. Although not directly related to the Statement of Work, the lead author, Cynthia Burroughs, Ph.D., was awarded a HBCU Faculty Scholar Research Award for this poster. Dr. Burroughs is on the faculty at a neighboring undergraduate institution, Philander Smith College, and has experience with mouse mammary glands. She works in the laboratory full-time during the summer and part-time during the academic year. This collaboration represents a Career Development effort that was especially relevant to procuring additional research support for the laboratory from the State of Arkansas Breast Cancer Research Program. Dr. Burroughs successfully competed for an award from this program and will assist in the planned animal experiments. In September 2000, I was appointed director for the Arkansas Cancer Research Center confocal microscope laboratory based on my expertise in digital light microscopy. Work from my laboratory was also presented in March 2002 at the California Breast Cancer Symposium. I reviewed research grant applications and participated in the pathogenesis review panel of the California Breast Cancer Research Program.

Bibliography of reports and presentations supported by this award:

1. Shank B.B., H.S. Wiley, and **R.C. Kurten** (2001) Structural and functional characterization of the human gene for sorting nexin 1 (SNX1) DNA and Cell Biology 20(5) 287-296.
2. **Kurten, R.C.,** B.B. Shank, A.D. Eddington (2000) Identification and characterization of the SNX1 gene. DoD Breast Cancer Research Program Era of Hope Meeting.
3. Burroughs, C.D., R.D. Smith, M.E. McIntire and **R.C. Kurten.** Intracellular pools of receptors modulate the kinetics of receptor down-regulation. Proceedings of the American Association of Cancer Research 41:740, 2000.

Individuals receiving pay from this award:

Richard C. Kurten Ph.D. - Principle Investigator
Cythnthia Burroughs Ph.D. - Collaborator
Brian B. Shank M.S. - Research Technician
Anthony Eddington M.S. - Graduate Student

CONCLUSIONS

We have cloned and characterized the human gene for SNX1. The SNX1 gene is alternatively spliced and characterization of its structure will aid in understanding the regulation,

and significance of the alternative splices. We have also identified the functional promoter in transfection experiments and find that it contains features characteristic of a house keeping gene. The SNX1 genomic clone and subclones that we now have in hand have also provided useful reagents for gene knockout studies to better understand the function of SNX1 inside cells. A graduate student, Parag Chowdhurry, has constructed a targeting vector and is attempting to use it to knock out the SNX1 gene in HEK 293 cells. Our determination of the chromosomal localization of SNX1 could facilitate the potential assignment of SNX1 as a disease locus identified in human genetic mapping studies. Currently, no diseases map to the SNX1 locus. Given the large size of the introns in the SNX1 gene, we decided not to pursue our first choice of replacing coding exons and intervening sequences of the whey acidic protein gene with SNX1 genomic sequences. Instead, we inserted a SNX1 cDNA fragment to generate our minigene for injection into mouse oocytes.

Over the last several years, projects in my laboratory and in other laboratories (3-5) have led to questions regarding the specificity and the utility of SNX1 in down-regulating EGF receptor expression. In part, these reservations may have arisen from our use of overexpression strategies to study SNX1. For this reason, transgenic mouse production was temporarily delayed while we evaluated the problem. As we proceeded with the transgenic mouse production efforts, we encountered substantial mortality in the pups, indicating that ectopic SNX1 overexpression might be lethal. Although we eventually generated a transgene positive female, we were unable to generate transgenic progeny and do not yet know if SNX1 is expressed in the mammary glands.

We think that our plans for gene knockouts and dominant negative constructions will provide additional support for the functional identification of SNX1 as a down regulator of EGF receptors using experimental approaches distinct from overexpression. We now have evidence that SNX1 exists in a multi-protein complex consisting of oligomers of SNX1, SNX2 and possibly 3 additional proteins. The fact that SNX1 is part of a multi-protein complex may account for variability problems in our cultured cell assays. In any event, the conservation of function and biochemical properties between SNX1 and its yeast homologue, Vps5p (6), make it clear that SNX1 has a role in endosomal membrane trafficking. With our continued studies in cell culture models and transgenic animals, we expect to make progress in understanding the role of SNX1 in cell proliferation and EGF receptor trafficking the mammary gland.

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APPENDICES

Appendix 1 - Figure Legends

Figure 1. Chromosomal localization of SNX1 and SNX2. Fluorescence in situ hybridization of P1 genomic DNA clones isolated using SNX1 and SNX2 cDNA fragments. Biotinylated DNAs corresponding to genomic clones for SNX1 (3E2) and SNX2 (B100-3) hybridized with efficiencies of 87.5% and 85%, respectively. Two additional P1 clones isolated with the SNX1 cDNA (6K1 and 6K7) yielded similar results.

Figure 2. Structural Organization of the SNX1 Gene and Correspondence to the SNX1 cDNA. The SNX1 gene map was generated using data generated in our laboratory and by the human genome sequencing project. The map is based on an insert containing 4.5 kb 5'-flanking DNA, a 43 kb 6K1 fragment that contained all the SNX1 coding DNA and approximately 16 kb 3' flanking DNA. The dark bars mark the locations of 15 identified exons. Below the genomic map is a cDNA map containing the SNX1 open reading frame within a 1974 bp cDNA. The location of a PX domain, (PX), several regions predicted to form coiled-coils (CC1, CC2, CC3) and a receptor binding domain (RDB) are superimposed on the open reading frame. The numbered boxes below the open reading frame indicate the regions of the cDNA encoded by the 15 exons. Several alternatively spliced (skipped) exons are designated Δ.

Figure 3. Map of plasmid pWAP 5'&3'. This plasmid was constructed by cloning PCR fragments corresponding to the 5' (WAP -949 to -7) and the 3'-end (WAP 3' PCR Product) of the rat WAP gene. The unique EcoRI site separating the WAP 5'-end and 3'-end fragments was used for insertion of the SNX1 cDNA.

Figure 4. Map of the plasmid from which the linearized SNX1 minigene is derived. Plasmid pWAP-SNX1cDNA was generated as described. The ethidium bromide stained agarose gel shows the 3375bp SalI-NotI linearized minigene fragment that was gel purified for use for microinjection into mouse oocytes to generate transgenic mice. The size was estimated by comparison to BstEII digested λ DNA. Also included on the agarose gel is a 4078bp WAP-GFP-

SNX1 minigene that we generated for potential use in transgenic animal generation using the green fluorescent protein as a marker for SNX1 expression.

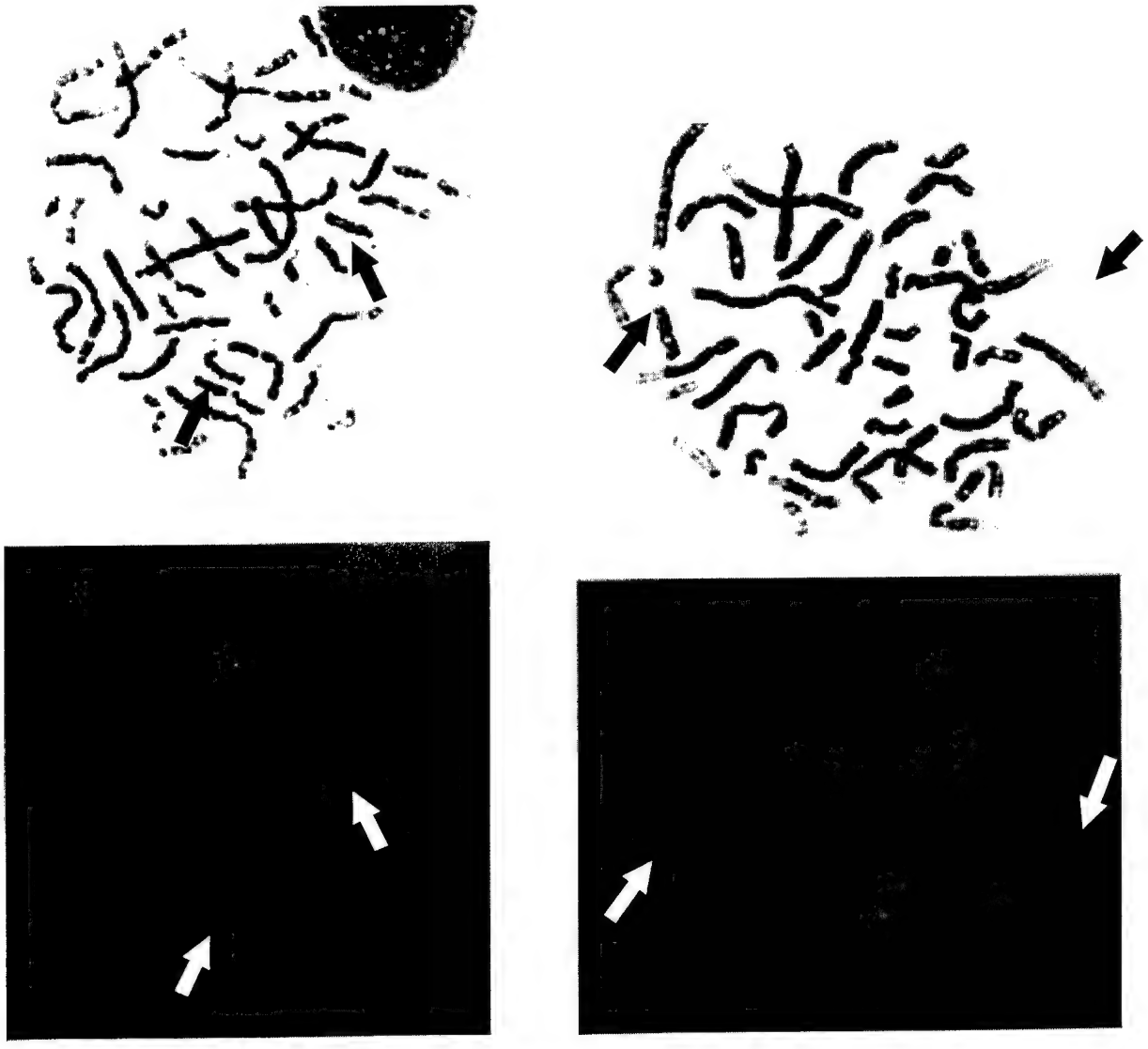
Figure 5. Specificity and sensitivity of pWAP-SNX1 transgene specific amplimers in the presence of FVB mouse tail genomic DNA. PCR reactions were performed in a volume of 25µl with the indicated amounts of pWAP-SNX1 transgene and/or FVB mouse genomic DNA. One primer was specific for human SNX1 and the other was specific for rat WAP DNA. Assuming that there are $1.6x10^5$ copies of transgene per 100ng genomic DNA, this assay will easily detect the specific transgene in mouse tail DNA.

Figure 6. Identification of a WAP-SNX1 minigene positive female mouse by PCR analysis of tail DNA. PCR reactions were performed in a volume of 50µl using FVB mouse genomic DNA samples and pWAP-SNX1 minigene-specific (A, B) or human SNX1-specific primers (C). The assay depicted in panel A identifies a single mouse (mouse 300) in a litter born March 19, 2001 that is positive for the microinjected pWAP-SNX1 minigene. However, the signal is very weak, and the positive control reaction is smeared. Panel B is a titration of the PCR reaction using the indicated number of copies of the minigene in the presence of FVB genomic DNA that demonstrates that the primers are working properly. To confirm that mouse 300 is positive for the WAP-SNX1 transgene, a different set of PCR primers were therefore used (C). Panel C is a PCR analysis of the same mouse genomic DNAs as in panel A using primers diagnostic for the human SNX1 gene. As was the case in panel A, mouse 300 is positive for the presence of human SNX1 DNA. Using this primer pair, the 5000 minigene copy control sample also yielded the expected product. Thus, mouse 300 is positive for the WAP-SNX1 minigene.

Figure 7. Western Blot Characterization of Affinity Purified Anti-SNX1 (Batch 2) using HeLa cytosols containing HA-SNX1. Cytosols were prepared from HeLa cells overexpressing a HA epitope-tagged SNX1. The indicated volumes of the cell extracts were electrophoresed on SDS-PAGE gels, transferred to PVDF membranes and blotted with affinity purified antibodies raised against the peptide CKYLEAFLPEAKAIS. Antibody preparations from two different rabbits (5551/2 and 5552/2) were analyzed either fresh or following aliquoting and a cycle of freezing and thawing. From this analysis, we conclude that 5551/2 is the more sensitive and specific antibody preparation and that both preparations are stable to freeze-thaw.

Figure 8. Scatchard plots of saturation binding assays to measure EGF receptor numbers in cultured breast cell lines. Immortilized (HB2) and tumor cell lines (MCF7v and HBT22) derived from human breast tissues were cultured to 70% confluence and serum starved overnight. Cultures were chilled to 4C and incubated with ^{125}I-EGF in the absence or presence of increasing concentrations of mouse EGF for 4 hours. After washing, the cells were dissloved in 1N NaOH and the radioactivity measured in a gamma counter. The data were reduced by Scatchard analysis assuming a single class of ligand binding sites. Based on these measurements, we calculate that the MCF7v line (closed squares) expresses ~380,000 receptors per cell, the HB2 line (open squares) expresses ~77,000 receptors per cell and that the HBT22 line (triangles) is devoid of EGF receptors.

Figure 1



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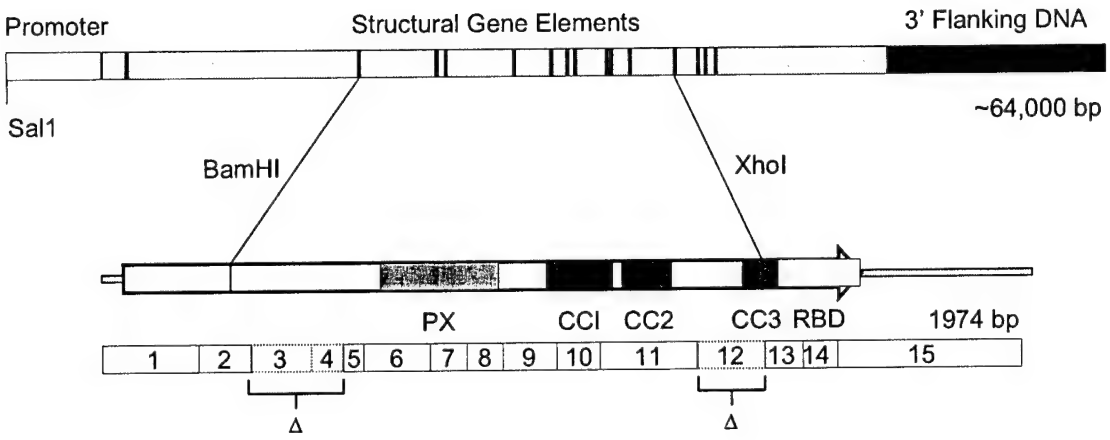
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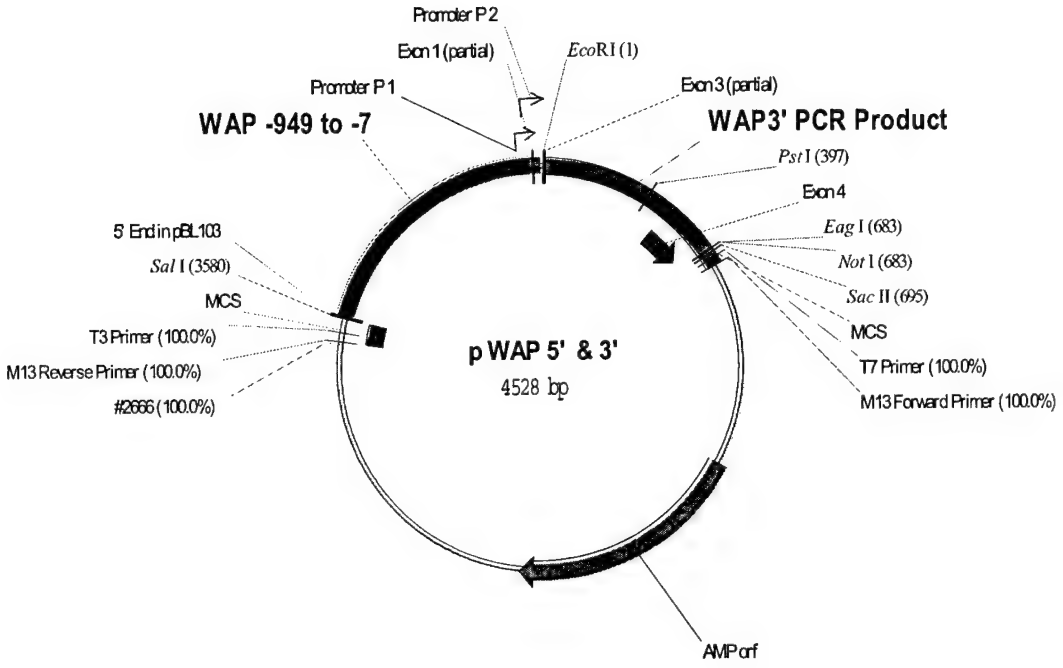
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Figure 4

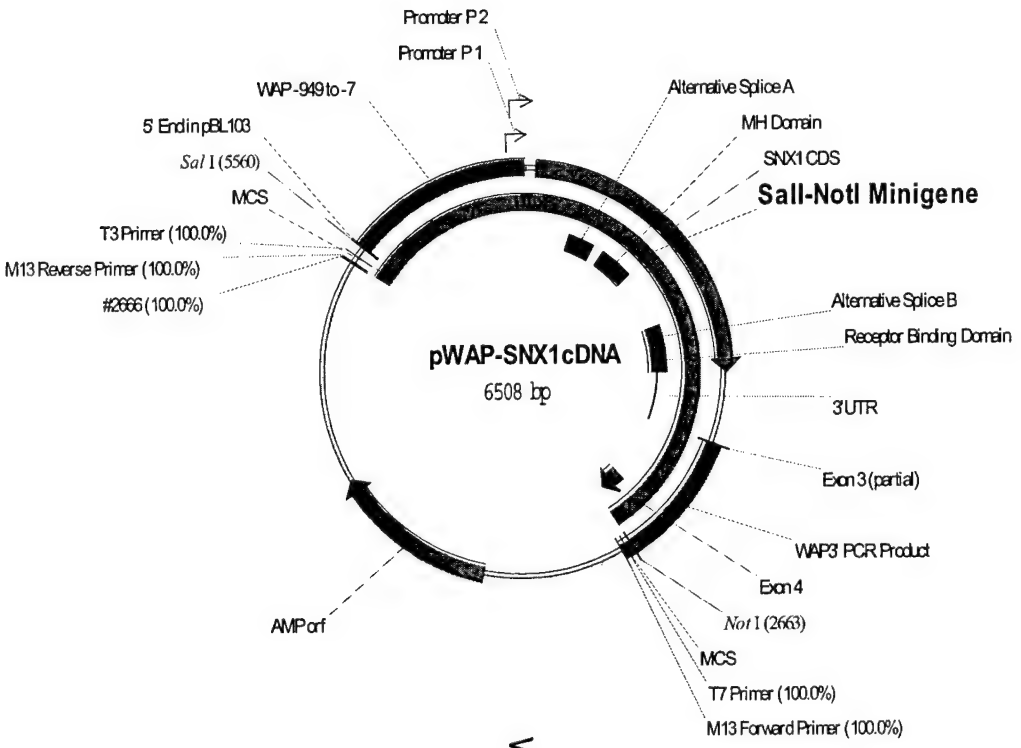
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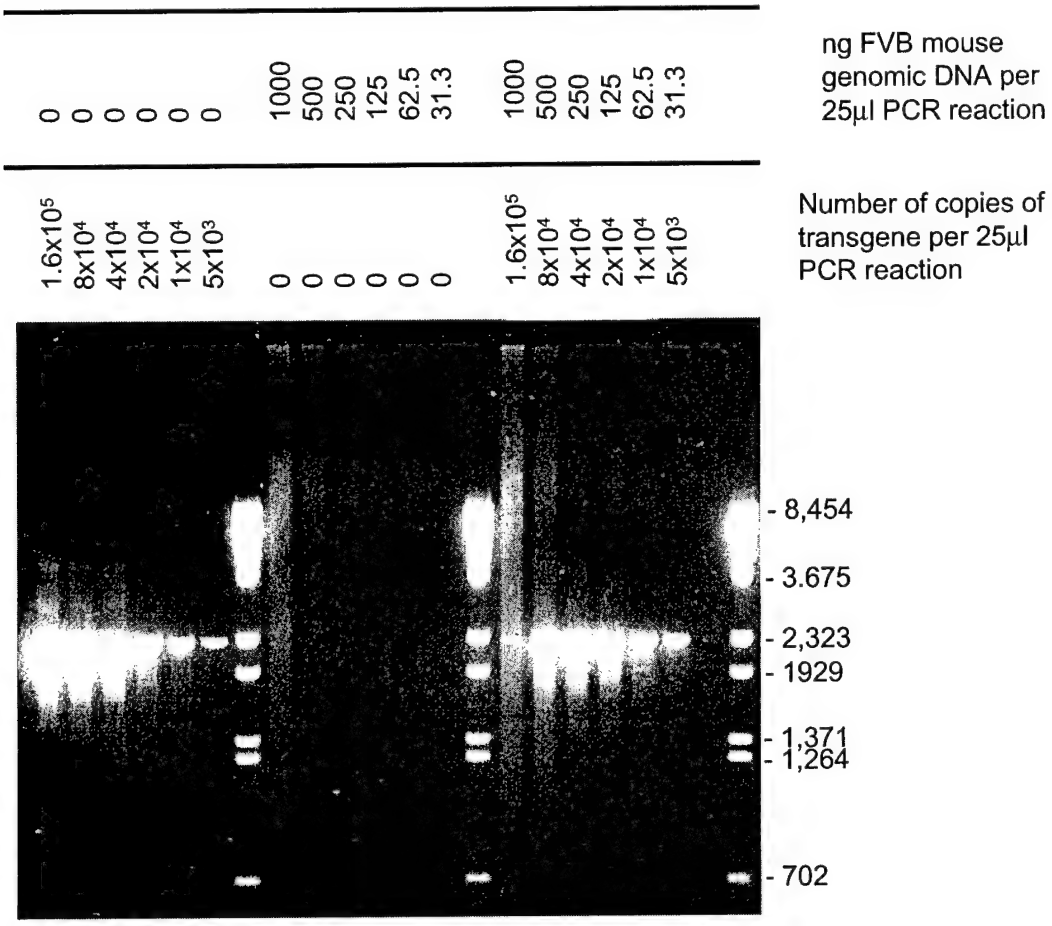
Figure 5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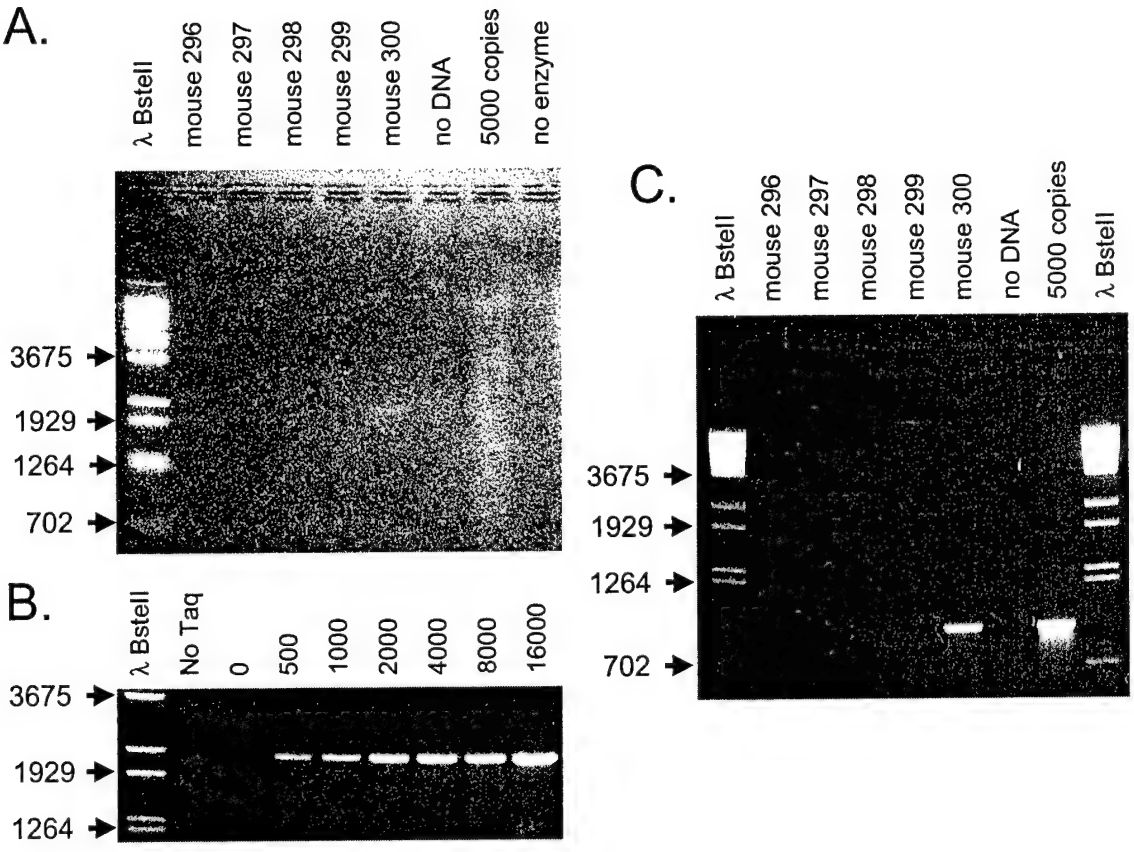
4x10^4
2x10^4
1x10^4
5x10^3
- 8,454
- 3.675
- 2,323
- 1929
- 1,371
- 1,264
- 702

Figure 6


A.
λ BstEII
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mouse 297
mouse 298
mouse 299
mouse 300
no DNA
5000 copies
no enzyme
3675
1929
1264
702
B.
λ BstEII
No Taq
0
500
1000
2000
4000
8000
16000
3675
1929
1264
C.
λ BstEII
mouse 296
mouse 297
mouse 298
mouse 299
mouse 300
no DNA
5000 copies
λ BstEII
3675
1929
1264
702

Figure 7

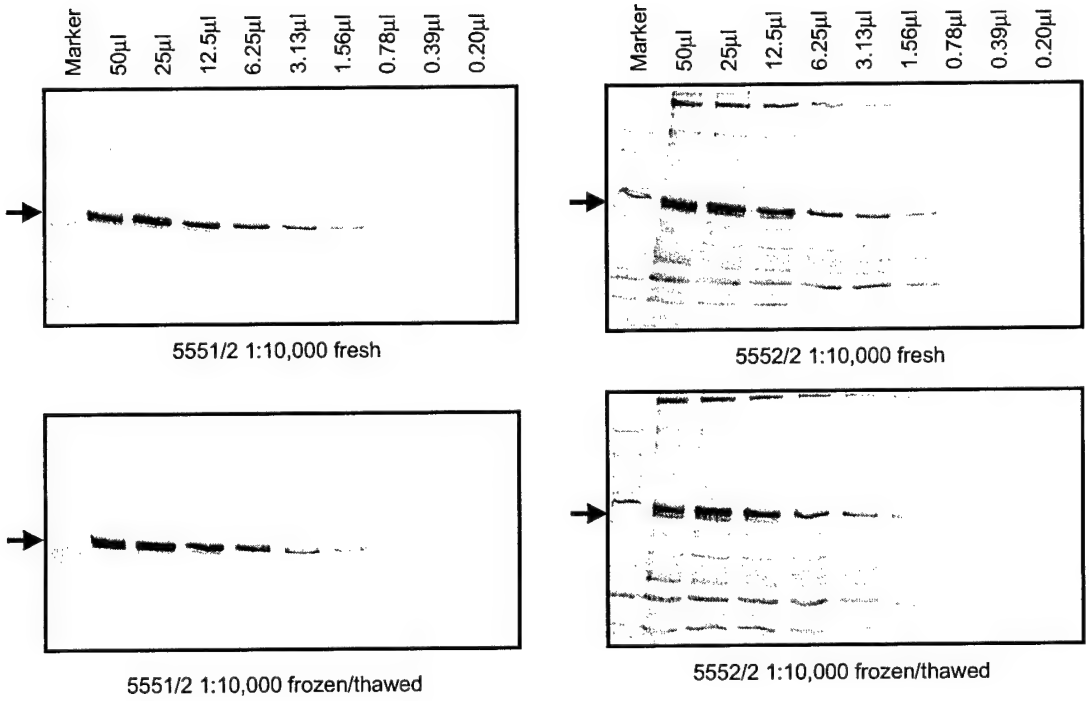
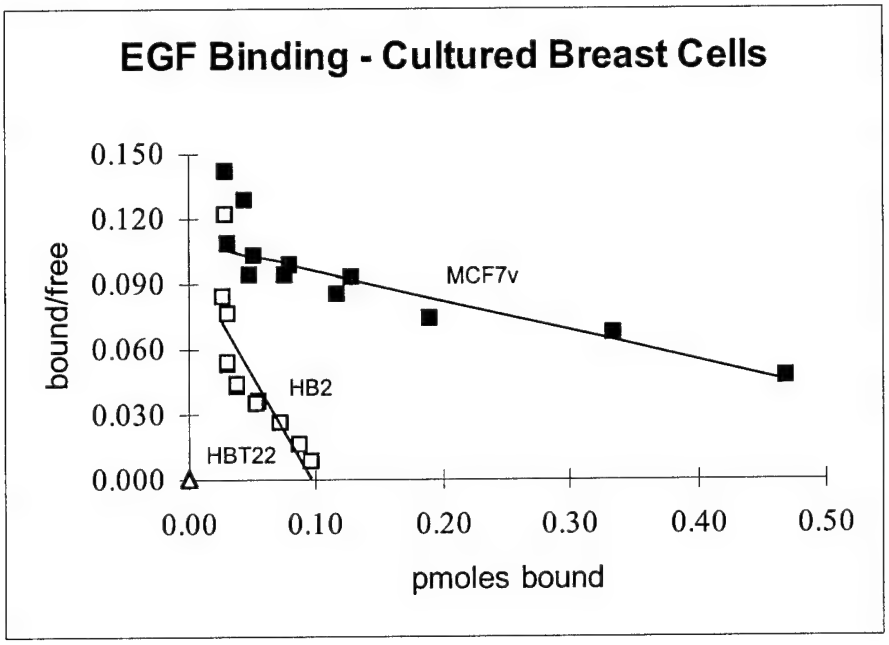

Marker
50µl
25µl
12.5µl
6.25µl
3.13µl
1.56µl
0.78µl
0.39µl
0.20µl
5551/2 1:10,000 fresh
5552/2 1:10,000 fresh
5551/2 1:10,000 frozen/thawed
5552/2 1:10,000 frozen/thawed

Figure 8


EGF Binding - Cultured Breast Cells
bound/free
0.150
0.120
0.090
0.060
0.030
0.000
MCF7v
HB2
HBT22
0.00
0.10
0.20
0.30
0.40
0.50
pmoles bound

Appendix 2 - Abstract of poster presentation

Poster presented at the DoD Breast Cancer research Program Era of Hope Meeting June 8-12, 2000 Atlanta, Ga.

Identification and Characterization of the Human SNX1 Gene. Richard C. Kurten, Bryan B. Shank and Anthony D. Eddington, Department of Physiology and Biophysics, University of Arkansas for Medical Sciences

Excessive activation of growth factor receptors can lead to the unrestrained cellular proliferation characteristic of tumors. Our objective is to determine if SNX1, a protein involved in intracellular membrane trafficking, can be used to downregulate EGF receptors in mammary gland, thereby attenuating EFG receptor dependent proliferation. Our approach is to characterize the human gene for SNX1 and to generate transgenic mice overexpressing human SNX1 in the mammary glands. Three bacterial artificial chromosomes (BAC) containing the human SNX1 gene were isolated in collaboration with H. Steven Wiley (University of Utah School of Medicine). Fluorescence *in situ* hybridization was used to determine the chromosomal localization of SNX1 to human chromosome 15q22 and of SNX2 (a closely related protein) to chromosome 5q23. One of the BAC clones for SNX1, labeled 6K1, has been characterized by subcloning, restriction mapping and DNA sequencing. The complete SNX1 coding region lies within 55kb of genomic DNA as defined by sequencing and oligonucleotide hybridization and consists of at least 15 exons. Several of the intron/exon junctions correspond to sites for alternative splicing as previously defined in cDNA clones. Alternatively spliced SNX1 mRNAs were detected in a variety of tissues using reverse-transcriptase polymerase chain reactions but they were less abundant than the full-length SNX1 mRNA species. The first intron of SNX1 is 16.5 kilobases in length and therefore unsuitable for the production of a transgenic expression vector. We next adopted a cDNA approach that has been used successfully for stromolysin 1 (Sympson, et al., 1994, *J Cell Biol* 125(3):681-93) and IGF (Neuenschwander, et al. 1996, *J Clin Invest* 97(10):2225-32.). We obtained plasmid pbl103 containing bases -949 to +33 of the rat whey acidic protein (WAP) gene and plasmid pbTAPW3' containing 843 base pairs of WAP 3' sequence including the exons 3 and 4 and the 3' untranslated region from Jeffrey Rosen, Baylor College of Medicine, Houston, Texas. We used these plasmids to generate a WAP-SNX1 cDNA vector consisting of 943bp rat WAP 5' sequence and 820 bp WAP 3' sequence. pWAP-SNX1 was cleaved with SalI and NotI and the linearized minigene purified for mouse oocyte microinjection. To analyse for transgenics, we developed a set of amplimers and reaction conditions that detect 5000 copies of SNX1 transgene in the presence of 1.25 micrograms/ml FVB mouse tail genomic DNA. The production of transgenic animals with the vector we have prepared will allow us to test the utility of SNX1 for repressing EGF receptor dependent tumor growth in the mouse mammary gland.

The U.S. Army Medical Research and Materiel Command under DAPD17-98-1-8175 supported this work.

Appendix 3 - Manuscript in press

The attached manuscript is in press in DNA and Cell Biology.

Structural and Functional Characterization of the Human Gene for Sorting Nexin 1 (SNX1). Brian B. Shank, H.S. Wiley, and Richard C. Kurten

The aim of the present study was to identify the gene for SNX1 to evaluate the potential for tissue specific alternative splicing and to analyze the activity of the SNX1 promoter. The coding DNA for SNX1 is divided between 15 exons in 43 kb of genomic DNA located on human chromosome 15q22. Although SNX1 mRNA expression is widespread in human tissues, alternative splicing is thought to generate skipped exons in SNX1 cDNAs. Based on determination of the SNX1 gene structure and an analysis of SNX1 mRNAs in a variety of tissues using reverse-transcriptase polymerase chain reactions, we demonstrate that SNX1 mRNAs are alternativelly spliced. Exon skipped products were less abundant than full-length SNX1 mRNA species, but the ratio of skipped to full-length mRNA indicated that alternative splicing may be developmentally regulated in liver. Consistent with widespread mRNA expression, the SNX1 promoter was GC rich and lacked a TATA box, features characteristic of housekeeping promoters. SNX1 promoter activity was dependent on the presence of proximal promoter sequences that contain initiator elements and predicted binding sites for the transcription factors Sp1 and E2F. These findings indicate that regulation of SNX1 gene expression at the transcriptional level is likely minor. Rather, developmentally specific exon skipping provides a potential mechanism for regulating the activity of SNX1.

Appendix 4 - Abstract resulting in award for the first author

Poster presented at the 91st Annual Meeting of the American Association of Cancer Research April 1-5, 2000 San Francisco, CA.

INTRACELLULAR POOLS OF RECEPTORS MODULATE THE KINETICS OF RECEPTOR DOWNREGULATION. C. Burroughs, R. Smith, M. McIntire and R. Kurten. University of Arkansas at Pine Bluff, Pine Bluff, AR 70611; University of Arkansas School for Medical Sciences, Little Rock, AR 72205

Different distributions of erbB2 receptors have been documented in different cell lines. In some cell lines, most receptors are inside the cell whereas in others most of the receptor is external. We have examine the distribution of epidermal growth factor receptor (EGFR) and the related receptor erbB2 in a virus transformed cell line derived from normal breast tissue (HB2), in an MCF7 cell line, and in a variant cell line derived from MCF7. Each breast cell line was grown to 70% confluence in DMEM/F12 with 10% bovine calf serum and 1% antibiotics. Cells where washed with PBS and serum starved for 24hr in DMEM/F12, 0.01% BSA and 1% antibiotics. Cells were exposed to 100 nM EGF in DMEM/F12, 0.01% BSA and 1% antibiotics and terminated at 2,4,6,12 and 24 hours after treatment. EGF receptor and erbB2 expression was examined in detergent extracts by western blotting. HB2 and MCF-7 cells expressed modest amounts of both EGF and erbB2 receptors. Treatment with EGF for 2 hours efficiently down-regulated both EGF and erbB2 receptors. By contrast, the variant MCF7 cells expressed high levels of receptors and the total mass of receptors was not affected by treatment with EGF for 2 hours. However, when variant MCF7cells were treated for 12 or 24 hours, there was efficient down-regulation of both EGF receptor and erbB2.

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Structural and Functional Characterization of the Human Gene for Sorting Nexin 1 (SNX1)

BRIAN B. SHANK,[1] H.S. WILEY,[2] and RICHARD C. KURTEN[1]

ABSTRACT

The aim of the present study was to identify the gene for sorting nexin 1 (SNX1) to evaluate the potential for tissue-specific alternative splicing and to analyze the activity of the *SNX1* promoter. The coding DNA for *SNX1* was divided between 15 exons in 43 kb of genomic DNA located on human chromosome 15q22. Although SNX1 mRNA expression was widespread in human tissues, alternative splicing is thought to generate skipped exons in SNX1 cDNAs. By determination of the *SNX1* gene structure and an analysis of the mRNAs in a variety of tissues using RT-PCR, we demonstrated that SNX1 mRNAs are alternatively spliced. Exon-skipped products were less abundant than full-length SNX1 mRNA species, but the ratio of skipped to full-length mRNA indicated that alternative splicing may be developmentally regulated in the liver. Consistent with widespread mRNA expression, the *SNX1* promoter was GC rich and lacked a TATA box, features characteristic of housekeeping promoters. The promoter activity was dependent on the presence of proximal sequences that contained initiator elements and predicted binding sites for the transcription factors Sp1 and E2F. These findings indicate that regulation of *SNX1* gene expression at the transcriptional level is likely minor. Rather, developmentally specific exon skipping provides a potential mechanism for regulating the activity of SNX1.

INTRODUCTION

SORTING NEXIN 1 (SNX1) is a peripheral membrane protein that participates in the endocytic processing of internalized cell-surface receptors (Kurten *et al.*, 1996; Haft *et al.*, 1998). The SNX1 protein is a prototype for a family of molecules including the closely related SNX2 (unigene cluster Hs.11183; 61% identical) that forms a complex with SNX1 (Haft *et al.*, 1988). The SNX1 and SNX2 proteins each contain a phox homology domain, termed PX, that includes a polyproline motif typical of SH3 or WW domain binding proteins (Ponting, 1996). On the basis of the similarity in the region of the PX domain, at least 15 additional human SNX proteins have been identified, including one, SNX5, that binds the Fanconi anemia complementation group A protein. Although PX domains are often associated with events that involve the actin cytoskeleton, GTP-binding proteins, and phospholipid membranes (Domin *et al.*, 2000), little is known about the functional significance of this domain. The SNX1 protein also contains a carboxyl-terminal epidermal growth factor (EGF) receptor-binding domain (Kurten *et al.*, 1996) and several regions that are predicted to form coiled-coil domains. These amphipathic helices are involved in protein–protein interactions and are also found in SNAREs and other proteins that participate in intracellular membrane trafficking (Sollner *et al.*, 1993; Weber *et al.*, 1998; Skehel and Wiley, 1998).

In addition to related human proteins, *SNX1* cDNAs can be identified in a variety of other species, including mice (94% identical), worms (37% identity over 437 amino acids), and yeast (27% identical over 237 amino acids). The yeast homolog, Vps5p/Grd2p, is involved in trafficking the carboxypeptidase Y receptor between the *trans*-Golgi network and the vacuole (Horazdovsky *et al.*, 1997; Nothwehr and Hindes, 1997). Biochemical analysis indicates that Vps5p binds to additional yeast proteins to form a membrane coat complex (Seaman *et al.*, 1998) and that this complex is conserved in human cells (Haft *et al.*, 2000). This conservation of structure and of function in vesicular trafficking between human SNX1 and yeast Vps5

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complexes indicates that SNX1 is likely to be required for a conserved aspect of vesicular trafficking.

The biologic properties of human SNX1 have been analyzed largely in overexpression experiments using the cloned cDNA. How and where SNX1 and related proteins work in the endocytic pathway remains incompletely defined. The primary structure provides few clues about potential enzymatic activities, and expression of the protein appears ubiquitous. We cloned and characterized the *SNX1* gene to determine if regulation of transcription and transcript processing influenced the protein expression. Several forms of SNX1 have been identified in cDNA cloning experiments that appear to represent alternative splices (Haft *et al.*, 1998) and might lead to a modification of the protein's function. If this is the case, then an analysis of tissue-specific alternative splicing would identify appropriate model systems with which to better define and dissect function. Accordingly, we determined the intron–exon structure of the *SNX1* gene, established that the different forms of SNX1 do arise from alternative splicing, and analyzed alternative splicing in a variety of tissues. We also identified the *SNX1* promoter and characterized its functional activity in cultured cells. This promoter has properties of a housekeeping gene and is dependent on the presence of an SP1/E2F box for maximal activity.

MATERIALS AND METHODS

Isolation and characterization of the SNX1 *gene*

cDNA fragments corresponding to amino acid residues 7–483 of SNX1 were generated by the PCR using oligodeoxyribonucleotide primers 5′-GGCTGTAGCGCTTCGGAGAGACT and 5′-CTTTCTCAAACCGTATCACTTCT. Agarose gel-purified PCR products were labeled to high specific activity using a random-priming kit (Pharmacia, Piscataway, NJ) and [α^{32}P]-dCTP. These cDNAs were used to screen a P1 human genomic DNA library (Pierce and Sternberg, 1992). After confirmation of the clones by secondary screening, DNA from individual clones 3E2, 6K1, and 6K7 was isolated using a kit (Qiagen, Santa Clarita, CA) followed by cesium chloride purification.

Southern blotting of human genomic DNA and cosmid clone 6K1

Human genomic DNA was purchased from Clontech (Palo Alto, CA). Genomic DNA was digested with *Bam*HI, *Eco*RI and *Nhe*I at 30 ng/μl and ethanol precipitated, and 10 μg of DNA per lane was electrophoretically separated on a 0.7% agarose gel in 1 × TAE. Very dilute quantities of cosmid DNA clone 6K1 were also digested with *Bam*HI, *Eco*RI, and *Nhe*I and electrophoretically separated at 3.2 × 10^6 copies per lane. Separated DNAs were blotted onto a Magna Charge Nylon membrane (Micron Separations, Westborough, MA) and fixed to the filter by baking at 80°C for 2 h under vacuum (Maniatis *et al.*, 1982).

Blotted membranes were prehybridized at 60°C with 5× SSC, 50 mM sodium phosphate, pH 7; 5× Denhardt's solution, 1% SDS, and sheared heat-denatured salmon sperm DNA 100 μg/ml. Membranes were then hybridized at 60°C for 18 h with 5× SSC, 50 mM sodium phosphate, pH 7; 1% SDS, sheared heat-denatured salmon sperm DNA 50 μg/ml, and radiolabeled *SNX1* cDNA 3.7 × 10^6 cpm/ml. The DNA was radiolabeled to a specific activity of 1 × 10^9 cpm/μg using a random-primed DNA labeling kit from Boehringer Mannheim (Indianapolis, IN), and unincorporated nucleotides were removed using a Sephadex G-50 spin column (Boehringer Mannheim). Hybridized membranes were washed with a solution of 1× SSC, 0.1% SDS at 60°C, wrapped in plastic wrap, and exposed on XOMAT-AR X-ray film (Eastman Kodak Company, Rochester, NY) with an intensifying screen at −70°C for two days.

The conditions for hybridization of radiolabeled oligodeoxyribonucleotides to restriction enzyme-digested 6K1 cosmid DNA and 6K1 subcloned fragments bound to NitroBind nitrocellulose membranes (Micron Separations) were identical to those described above except that hybridization was done in the absence of salmon sperm DNA. Oligodeoxyribonucleotides (10 pmol) were radiolabeled with 167 μCi of 7000 Ci/mmol [γ^{32}P]-ATP from ICN (Costa Mesa, CA) and 1 U of T4 polynucleotide kinase (Epicentre Technologies, Madison, WI) in a 10-μl reaction mixture. Radiolabeled DNA was separated from unincorporated ^{32}P using Sephadex G-25 spin columns. Membranes were reprobed after removal of the radiolabeled probe from hybridized membranes by incubation with 0.25× Denhardt's solution, 2.5 mM Tris HCl, pH 8; 0.1 mM EDTA, pH 8; and 0.5 mM sodium pyrophosphate at 90°–95°C for 20 min.

Rapid amplification of SNX1 *5′ cDNA end*

Adaptor-ligated double-stranded colon cDNA was purchased from Clontech. Adaptor-specific PCR amplimers AP1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and AP2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′) were also supplied by Clontech. Approximately 0.5 ng of cDNA material was amplified with AP1 and an *SNX1* exon 2-binding amplimer (5′-GGAGCCATTGTTGATGGGAAGAAG-3′; SNX1-2891) that binds the sense strand 192 bp downstream of the translation start codon. A 50-μl reaction volume containing 200 μM each DTP, 0.2 μM each amplimer, 1× Expand Polymerase Buffer 1, and high-fidelity Expand Polymerase 0.04 units/μl from Boehringer Mannheim was heat denatured at 94°C for 1 min in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA). The reaction was thermocycled 26 times with cycles of 94°C for 30 sec, 57°C for 30 sec, and 68°C for 1 min. The amplified material was diluted 1000 fold and reamplified with AP2 amplimer and an *SNX1* exon 1-specific amplimer (5′-CCGGTGAAAATGTCCTCCCCCT-3′; SNX1-1401) using identical PCR conditions. A major band of approximately 200 bp was gel purified using a gel extraction kit (Qiagen, Valencia, CA) and cloned into the TA-cloning vector pCR2.1 from Invitrogen (Carlsbad, CA).

Creating a restriction and exon map for the 6K1 cosmid clone

The *Bam*HI, *Nhe*I, and *Xho*I fragments of the 6K1 clone were subcloned into pBluescript II KS+ or pMOB (Strathmann *et al.*, 1991). Restriction mapping and DNA sequencing of the 6K1 subclones, as well as oligodeoxyribonucleotide hybridiza-

tion of restriction enzyme-digested 6K1 DNA or 6K1 subclones blotted to nitrocellulose filters (Micron Separations), provided data for the assembly of a *Bam*HI, *Eco*RI, *Nhe*I, *Sal*I, and *Xho*I restriction map for the 6K1 clone.

Exon–intron junctions were defined by sequencing various 6K1 subclones with oligodeoxyribonucleotides specific for the human *SNX1* cDNA, followed by sequencing in the reverse direction with intron-specific oligodeoxyribonucleotides that bind to a region adjacent to an intron–exon junction. Positions of exons on the 6K1 restriction map were determined by DNA sequence analysis, restriction enzyme analysis, or size estimation of PCR products on agarose gels.

cDNA database searches were performed using the NCBI Blast tools (www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al.*, 1990). Sequence alignments were done using the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison WI) and the BLAST 2 Sequences tool. Sequence files were managed using Vector NTI (Informax, N. Bethesda, MD).

Fluorescence in situ *hybridization*

Clones 3E2, 6K1, and 6K7 were used to determine the chromosomal location of *SNX1* using fluorescence *in situ* hybridization. The P1 DNAs were labeled with biotin in a nick-translation reaction, hybridized with metaphase chromosomes, and then detected with Cy3-conjugated streptavidin (Jackson Immuno Research, West Grove, PA). Human metaphase chromosome spreads were prepared by standard procedures (Marchilli, 1980), and *in situ* hybridization was performed according to the procedure described by Pinkel and associates with modification (Pinkel *et al.*, 1986). Slide-fixed material was denatured in 2× SSC, 70% ethanol for 2 min at 70°C; dehydrated in washes of 70%, 85%, and 100% ice-cold ethanol; and hybridized in a mixture containing 200 ng of biotinylated probe in Hybrisol VI (Oncor, Gaithersburg, MD) and 10 μg of Cot1 DNA (GIBCO/BRL, Grand Island, NY) at 37°C. After overnight incubation, the slides were washed at a stringency of 2× SCC/50% formamide at 43°C and stained with Cy3 conjugate and counterstained with DAPI. The program Smart Capture (Vysis, Downers Grove, IL) was used to visualize metaphase chromosomes and to convert the DAPI image into a black and white G-banded image to facilitate band localization. At least 10 metaphase cells were used for chromosomal localization.

Northern blotting

Membranes containing electrophoretically separated human Poly(A)$^+$ RNAs (Clontech) were prehybridized at 37°C in buffer containing 750 mM NaCl, 40 mM NaH_2PO_4, 5 mM EDTA, 0.2% Ficoll, 0.2% polyvinylpyrolidine, 0.2% BSA, sheared salmon sperm DNA 100 μg/ml, 50% deionized formamide, 2% SDS, pH 7.4, in a roller bottle. Hybridization was under identical conditions with inclusion of gel-purified *SNX1* cDNA fragment 10^6 cpm/ml labeled in a random-priming reaction with [α^{32}P]-dCTP. The probe employed for SNX1 was complementary to mRNA encoding amino acid residues 77–522 and included 72 bp of the 3′ UTR. Hybridized membranes were washed to a final stringency of 0.1× SSC, 0.5% SDS at 44°C and autoradiographed.

Analysis of tissue-specific splicing

Dilutions (1×, 10×, 100×, 1000×) of a human cDNA panel prepared in a 96-well plate were screened using the PCR to define skipped exons in SNX1 mRNA. The skipping of exons 3 and 4 was analyzed using primers specific for exon 1 (5′-GGCTGTAGCGCTTCGGAGAGAC-3′; No. 1141) and for exon 10 (5′-TGCTCCTCACACTCTACCTCCTGG-3′; No. 3275). These primers yielded a 944-bp product for the intact cDNA and a 749-bp product for the exon-skipped cDNA. For exon 12 skipping, primers specific for exon 7 (5′-AAAGTGAAAGTTGGGAAGGAAG-3′; No. 3274) and exon 13 (5′-CTTCTTTTCGGACCACTGTTGA-3′; No. 3276) were used. These primers yielded a 772-bp product for the intact cDNA and a 628-bp product for the exon-skipped cDNA. Polymerase chain reactions were performed using 1× *Taq* polymerase buffer (Stratagene, La Jolla, CA), 200 μM each dNTP 0.2 μM each oligodeoxyribonucleotide, and Taq2000 0.025 U/μl (Stratagene) in 96-well RAPID-SCAN plates containing lyophilized cDNA (Origene Technologies, Rockville, MD) using a PTC-100 Programmable Thermal Controller with a heated lid (MJ Research). Polymerase enzyme activity was reversibly blocked prior to thermocycling by addition of TaqStart antibody to the PCRs as recommended by the manufacturer (Clontech).

Polymerase chain reaction preparations containing oligodeoxyribonucleotides 1141 and 3275 were heated to 94°C for 2 min; thermocycled 35 times at 94°C for 45 sec, 60°C for 30 sec, and 72°C for 1 min 30 sec; and incubated at 5 min at 72°C. The thermocycling conditions for PCRs containing oligodeoxyribonucleotide primers 3274 and 3276 were identical except that the annealing temperature was 55°C instead of 60°C.

SNX1 *promoter analysis*

Fragments of *SNX1* containing 5′ flanking sequences were inserted into a modified promoterless firefly luciferase plasmid pGL3-Basic (Promega, Madison WI) 19 bp 5′ of the luciferase methionine initiator codon. The plasmid was modified by disrupting the *Bam*HI and *Sal*I sites by Klenow fill-in of the *Bam*HI and *Sal*I restriction site 5′ overhangs, followed by replacement of the *Kpn*I-*Nco*I multicloning site region with the *Nhe*I-*Sty*I multicloning site region of pEGFP-N1 (Clontech). Promoter activity was measured following transfection of African green monkey kidney cells (CV1) using the calcium phosphate method (Graham and Eb, 1973). Cells were cultured in DMEM-F12 medium supplemented with 5% calf serum and antibiotics in a 5% CO_2 incubator at 37°C. Thirty-one hours after transfection, cell lysates were prepared, and luciferase activity was measured using a dual luciferase kit (Promega) and a luminometer. Transfection efficiency was normalized by measuring the activity of *Renilla* luciferase expressed from a cotransfected plasmid, pRL-SV40.

RESULTS

SNX1 *gene structure*

Three bacterial artificial chromosomes containing the human *SNX1* gene were isolated by hybridization with a radiolabeled

SNX1 cDNA fragment corresponding to amino acid residues 7–483. Clone 6K1 contained the entire coding region, as shown by hybridization with 5′ and 3′ cDNA probes, and was characterized in detail. The pattern of hybridization of *Bam*HI-digested 6K1 with a *SNX1* cDNA probe was identical to that observed for *Bam*HI-digested human placental genomic DNA (Fig. 1), indicating that the structural features of the *SNX1* gene were accurately preserved in the cloned DNA. The hybridization patterns obtained using *Eco*RI and *Nhe*I were similar but not identical, indicating that restriction sites flanking the cloned gene were absent from the endogenous gene. Placement of these differences in the 5′ flanking region of the 6K1 clone was confirmed by blot hybridization with a *Hin*dIII-*Pst*I probe fragment from 6K1 containing the translational start site and hence the 5′ end of the cDNA. Again, the *Bam*HI digests gave identical hybridization patterns for both the endogenous and the cloned gene when hybridized with the *Hin*dIII-*Pst*I probe, which spans a *Bam*HI site immediately upstream of exon 1. By contrast, only a single hybridizing fragment was observed for *Eco*RI and *Nhe*I digests. Given the known 3′ boundary of the *Eco*RI and *Nhe*I fragments, this places a lower limit of ~7.5 kb on the amount of potential *SNX1* promoter sequences in 6K1. Sequence analysis confirmed the presence of more than ~4.5 kb of *SNX1* promoter sequence by identification of a *Sal*I site that is also present 4460 bp upstream of a 5′-RACE-predicted transcription start site in two working draft sequences of human genomic

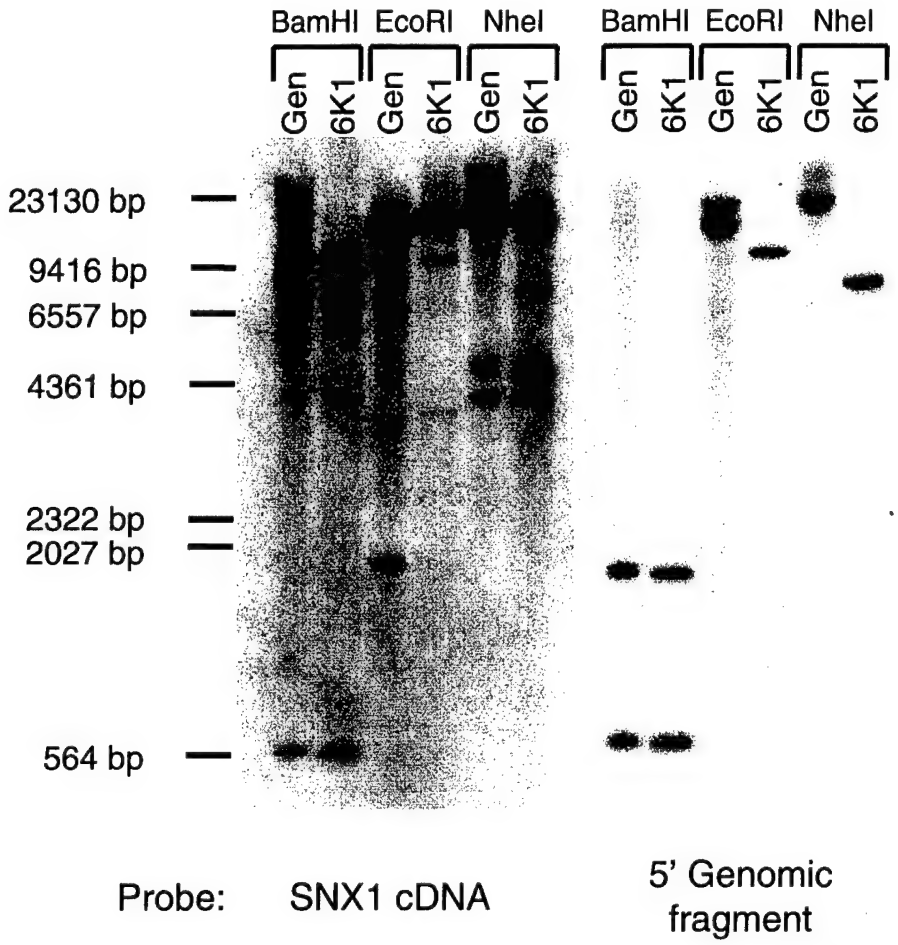

FIG. 1. Southern blot analysis of endogenous and cloned *SNX1* gene. Human genomic DNA and SNX1 genomic clone 6K1 were digested with restriction enzymes and analyzed by Southern blotting using an *SNX1* cDNA probe or a 5′ genomic fragment from 6K1 (*Hin*dIII-*Pst*I) that spans a *Bam*HI site immediately upstream of exon 1. The identical hybridization of *Bam*HI-digested genomic and 6K1 DNA with the *SNX1* cDNA probe indicates faithful cloning of all of the coding regions of the human *SNX1* gene. The digestion pattern is identical to that predicted on the basis of our map of the *SNX1* gene. Two of the eight *Bam*HI fragments within the *SNX1* gene that failed to hybridize (1669 bp and 8620 bp) are located within the 16-kb intron 1; hence, they should not be recognized by the cDNA probe. The use of the 5′ genomic fragment indicates that a 1.7-kb *Bam*HI fragment is upstream of and adjacent to the 563-bp *Bam*HI fragment containing exon 1 for both genomic and 6K1 DNAs. The 5′ genomic fragment hybridizes with *Eco*RI and *Nhe*I fragments in the 6K1 clone that are smaller than those for the endogenous *SNX1* gene. This result indicates that sequence differences between 6K1 and endogenous *SNX1* occur on *Nhe*I and *Eco*RI fragments containing exon 1. Sequence and restriction mapping analysis of a subcloned 5-kb *Sal*I-*Eco*RI fragment containing exon 1 indicates that the 6K1 genomic clone contains at least 4460 bp of 5′ flanking DNA.

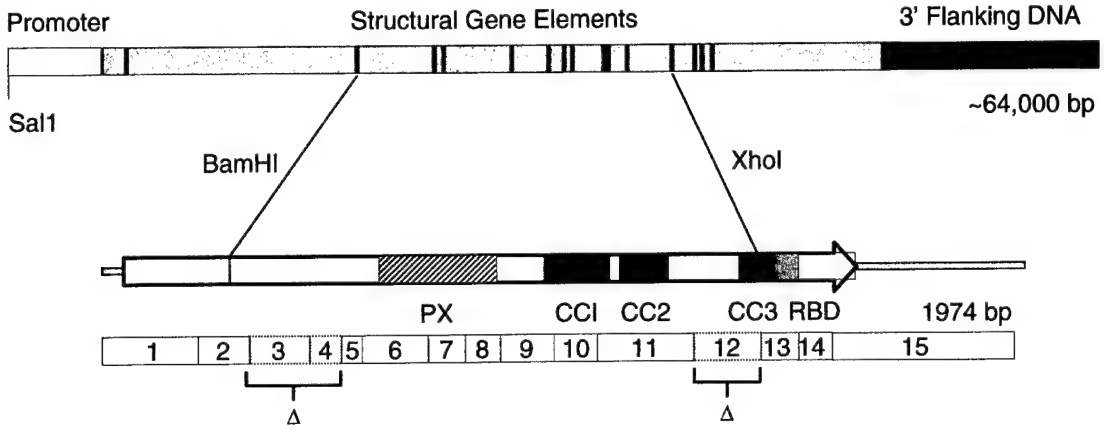

FIG. 2. Structural organization of the *SNX1* Gene and correspondence with the *SNX1* cDNA. The *SNX1* gene map was generated as described in the text using data obtained in our laboratory and confirmed by the Human Genome Project. The map is based on an insert containing 4.5 kb of 5′ flanking DNA, a 43-kb 6K1 fragment that contained all the *SNX1* coding DNA, and approximately 16 kb of 3′ flanking DNA. The dark bars mark the locations of 15 identified exons. Below the genomic map is a cDNA map of the *SNX1* ORF within a 1974-bp cDNA. The location of the PX domain (PX), several regions predicted to form coiled-coils (CC1, CC2, CC3), and a receptor-binding domain (RDB) are superimposed on the ORF. The numbered boxes below the ORF sequence indicate the regions of the cDNA encoded by the 15 exons. Several alternatively spliced (skipped) exons are designated Δ.

DNA (AC017098, AC024631). Thus, clone 6K1 contains both the promoter-enhancer elements and the structural elements of the human *SNX1* gene.

On the basis of analysis of clone 6K1, the structural gene for human SNX1 consists of 15 exons distributed within 43 kb of genomic DNA (Fig. 2). The average exon size was 128 bp, and the size ranged from 44 bp for exon 5 to 451 bp for exon 15. The introns ranged in size from 199 to ~16,000 bp (intron 1). The intron–exon junctions (Table 1) compiled with the GU-AG rule (Mount, 1982; Jacob and Gallinaro, 1989): 13 of the 14 5′ intron borders began with GU and 14 of 14 3′ intron borders ended in AG. Exon 15 contained the polyadenylation signal AATAAA (Proudfoot and Brownlee, 1976) at positions 44605 and 49694. To define the end of exon 15, the 3′ flanking *SNX1* genomic sequence was used to search the human EST database. There were greater than 50 ESTs that diverged 18 to 20 bp downstream of the AATAAA at position 44605. No ESTs extended to the AATAA at position 49694, nor did any ESTs align with the *SNX1* genomic clone anywhere downstream of 44625.

cDNA and EST cloning indicated that exon skipping may occur during SNX1 pre-mRNA processing. Our analysis of the *SNX1* gene structure demonstrated that exons 3 and 4 are deleted in

TABLE 1. INTRON–EXON JUNCTIONS IN *SNX1* GENE

Exon	Exon size	Splice acceptor junction		Splice donor junction		Intron size
		Intron	Exon	Intron	Exon	
1	174			— gccgcggugguc	gugaguuugcac	16513
2	112	cuuuguuuucag	aguaaacaucag	— aucucuuugcag	gcaaguuuggac	5498
3	128	acuucauuuuag	augccacagugg	— accuaugaggag	gugaggaucugu	545
4	67	uucuugaaaaag	cuagaggaagaa	— cugagaagauag	guaggugguucu	4857
5	44	guucucucacag	gggaugguauga	— guuacaacacag	gugaguccaggu	2680
6	142	ccugucccccaag	acaagcuuacca	— agagccucauag	guaaugccugug	998
7	79	auuccauucuag	ggaugacaaaag	— cgcuuuagaaag	guaagugccaug	479
8	76	cuuaccuuuuag	guaccuucagag	— gaaaaagaagag	guuaguauucag	2210
9	114	uuggucuuguag	cugccacgugcc	— gaaucagacauu	gugaguagcccu	199
10	94	uuuguguuucag	ugguuugaggag	— accauaggaaag	guaacaagcucu	1250
11	206	uccuccuuccag	agcuagcgcuga	— gccauaguccgc	guaagcuucugu	3048
12	144	ccacccccacag	gcugccuucgac	— gagauccucgag	gugaguccacug	1530
13	81	cuuucuucccag	ugggagucucgg	— auacgguuugag	gugagauagaaa	451
14	72	cuugucuccuag	aaagagaaaucc	— ucacagcagcag	guauguaaguug	636
15	451	uacucacagcag	cagcuggcaaag			

The coordinates for exon boundaries in our genomic clone and cDNA sequence map are listed for the 15 exons that were identified. Sequences of the corresponding intron–exon and exon–intron junctions are also listed. The gaps indicate the point of cleavage for processing, and the dashes represent the unlisted exon sequences.

SNX1A (Haft *et al.*, 1998) (gi3152941 and gi519376). We also cloned a HeLa cDNA that contained an exon 12 deletion. A BLAST search of the NCBI human EST database identified at least two additional examples of alternative splicing: one (gi5857289) contained a deletion of exons 13 and 14, and another (giA1094405) contained a deletion in the 3′ UTR within exon 15.

To determine if the exon 3, 4 and exon 12 skipping events were biologically relevant, mRNA from a variety of tissues was analyzed by Northern blotting to identify the smaller mRNAs that would arise from these events. The *SNX1* cDNA hybridized with an abundant and widely expressed 2-kb transcript and, to a lesser extent, with an 8-kb transcript (Fig. 3), but no smaller mRNAs were detectable. The larger transcript could be a precursor RNA or another SNX RNA hybridizing with the probe. When the same blot was probed with SNX2, the most closely related SNX family member identified to date, the 8-kb transcript, was not detected (data not shown), indicating that it is most likely a precursor RNA. The inability to detect smaller mRNAs indicates either that the exon-skipping events are rare or that the skipped exons are so small (195 and 144 bp) that the distinct species were not resolvable on the blot from the 2-kb species containing all the exons in the Northern blots.

To discriminate between these two possibilities, cDNA libraries prepared from a variety of human tissues were analyzed in PCRs to identify products corresponding to the exon-skipped species (Fig. 4). For primers, pairs of oligodeoxyribonucleotides specific for regions containing exons 1–10 or exons 7–13 were used. In the event of exon skipping, a PCR product ($SNX1_{1,2\Delta5-10}$ or $SNX1_{7-11\Delta13}$) that is smaller than the non-skipped product ($SNX1_{1-10}$ or $SNX1_{7-13}$) will be produced. Species corresponding to both exon-skipping events were detected. Compared with $SNX1_{1,2\Delta5-10}$, $SNX1_{7-11\Delta13}$ was detected infrequently, indicating that exon 3, 4 skipping is more likely be biologically relevant. Although the non-spliced $SNX1_{1-10}$ transcript predominated in all tissues, the ratio of $SNX1_{1,2\Delta5-10}$ to $SNX1_{1-10}$ was highest in fetal liver. The $SNX1_{1,2\Delta5-10}$ form was barely detectable in mature liver, suggesting developmental regulation of this exon-skipping event. Interestingly, in at least four of the tissues analyzed (small intestine, thyroid gland, peripheral blood leukocytes, and bone

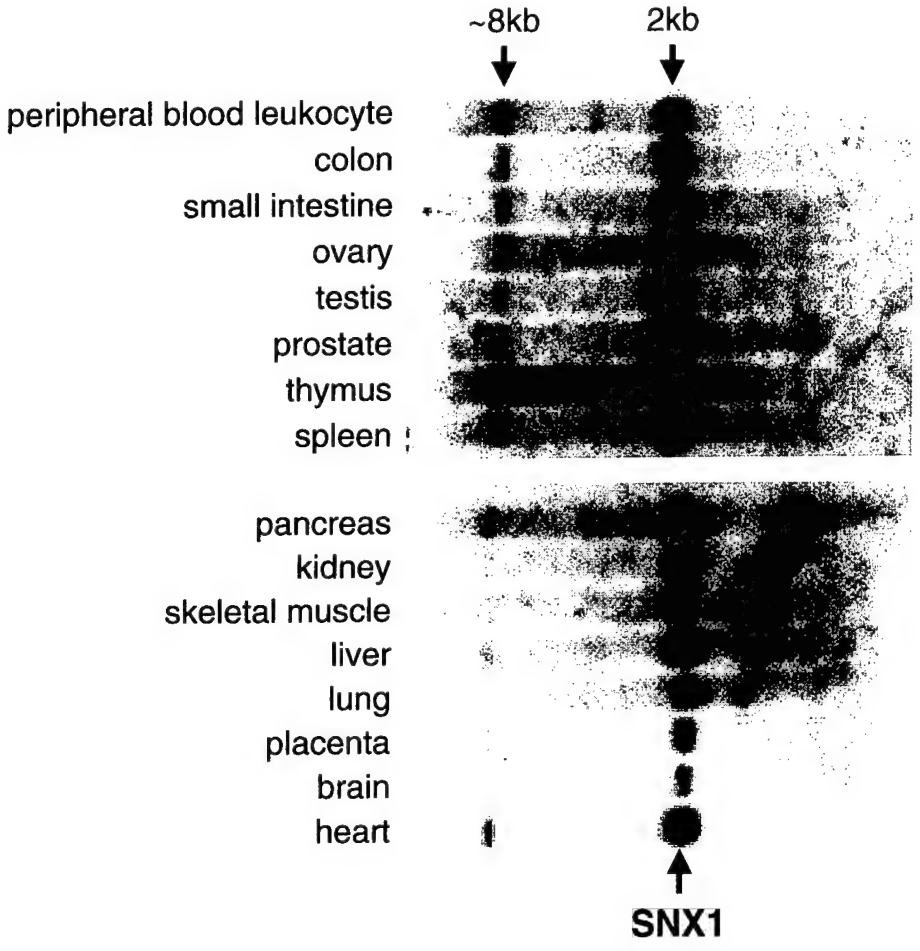

FIG. 3. Northern blot analysis of *SNX1*. Membranes prepared using 5 μg of electrophoretically separated poly(A)$^+$ RNA extracted from the indicated human tissues were hybridized at high stringency with a radiolabeled, gel-purified *Bam*HI-*Sal*I *SNX1* cDNA fragment. The sizes of the hybridizing bands were estimated by comparison with the positions of RNA size markers run on the same gels.

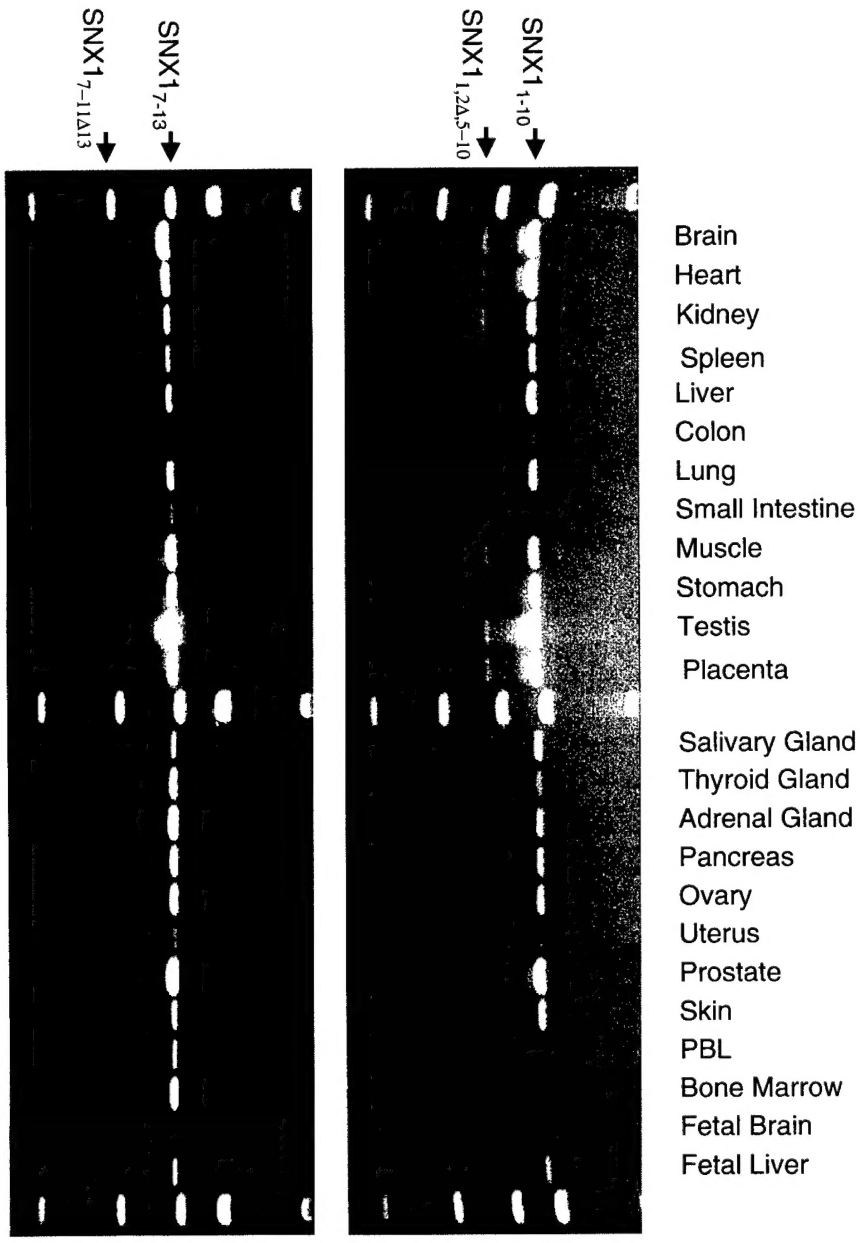

FIG. 4. Analysis of *SNX1* alternative splicing (exon skipping). Complementary DNA and EST cloning identified two alternatively spliced segments within the SNX1 mRNA. Alternative splicing in human tissues was analyzed in PCRs using a cDNA library panel and pairs of oligodeoxyribonucleotides specific for regions containing an exon 3, 4 deletion ($SNX1_{1,2\Delta5-10}$) or an exon 12 deletion ($SNX1_{7-11\Delta13}$). If the transcript is spliced, a smaller PCR product will be produced. With the exception of $SNX1_{1,2\Delta5-10}$ in fetal liver, the non-spliced transcripts were much more abundant than were the spliced transcripts in all tissues. Of the two deletions, $SNX1_{1,2\Delta5-10}$ occurred more frequently than did $SNX1_{7-11\Delta13}$. In four tissues (small intestine, thyroid gland, PBL, and bone marrow) there were substantially higher levels of the $SNX1_{7-11\Delta13}$ product than of the SNX_{1-10} product.

marrow), there were substantially higher levels of the $SNX1_{7-13}$ species than of the $SNX1_{1-10}$ species.

Identification of the SNX1 *promoter*

The genomic sequences 5′ of the *SNX1* protein-coding region were analyzed for promoter activity. First, the start site of gene transcription was determined by PCR cloning cDNAs generated in a primer extension reaction using human colon mRNA and RT. The longest PCR product that was identified mapped to a cytosine 15 bp upstream of the methionine initiator codon. We denoted this cytosine +1. This clone aligned with the mouse *SNX1* cDNA (gi5053009) and with a bovine EST (7032078), but there were no human clones that extended further upstream than the human colon cDNA RACE product. There were no TATA boxes in the *SNX1* promoter, but three potential tran-

scription initiators (Inr) (Javahery *et al.*, 1994) were detected that would initiate at −33, −70, and −184. There was a good identity (>90%) between the mouse cDNA, the bovine EST, and the human gene up to the Inr at −33. We conclude that this Inr is likely the most commonly used for the human *SNX1* gene. The assignment of this transcription initiation site is also consistent with the deduction based on restriction hybridization mapping that clone 6K1 should contain the *SNX1* promoter (see Fig. 1).

To analyze functional promoter activity, an *SNX1* promoter fusion (−1762 to +1) to firefly luciferase at the +1 site was constructed. Following transfection into CV-1 cells, promoter activity was easily detected and was higher than that driven by an SV40 promoter (Fig. 5). To control for transfection efficiency, expression was normalized to a contransfected *Renilla* luciferase gene. To map the boundaries of the *SNX1* promoter, a series of deletions were also analyzed for promoter activity. Progressive deletion from the 5′ end (−1762, −446, −159) revealed that as little as 160 bp (−159 to +1) retained 86% of the promoter activity. Promoter activity was absolutely dependent on this region because a 3′ deletion yielding −1762 to −160 was inactive. Within the 160 bp region, −100 to +1 and −159 to −100 each contributed about half of the promoter activity. Present within −100 and +1 are two Inr sequences at −33 and −70. Present within −159 to −100 are potential binding sites for the transcription factors Sp1 (three sites) and E2F (Sp1/E2F box). Internal deletions upstream of the Sp1/E2F box (−1762 to −446/−283 to +1 and −1762 to −446/−159 to +1) had little effect on promoter activity (data not shown). Thus, the minimal functional promoter for *SNX1* consists of the proximal 160 bp and is likely stimulated by Sp1 transcription factor binding. These features and the lack of a recognizable TATA box are characteristic of the genes for housekeeping proteins (Ishii *et al.*, 1985; Melton *et al.*, 1984; Reynolds *et al.*, 1984).

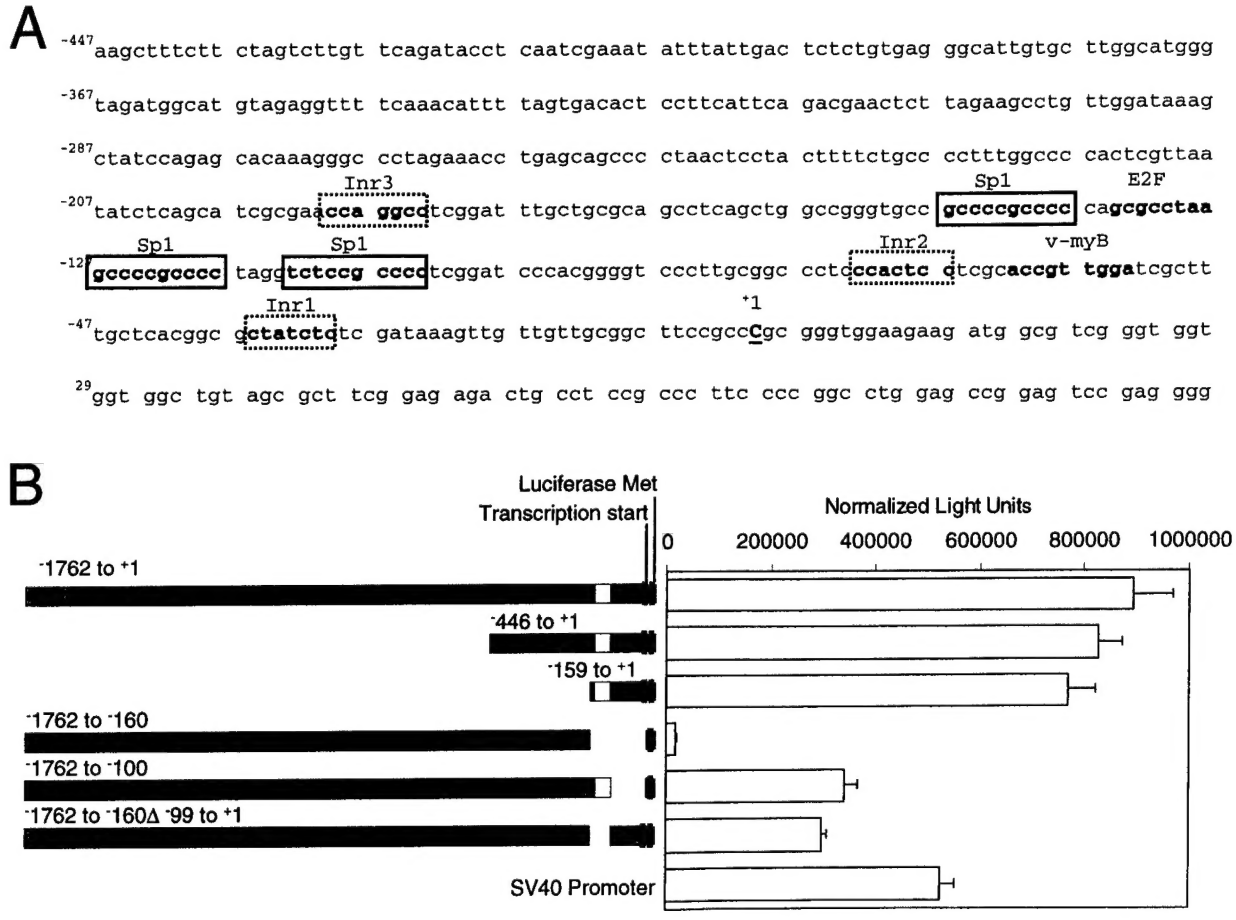

FIG. 5. Functional identification of the basal *SNX1* promoter. (**A**) The sequence of the proximal region of the *SNX1* gene promoter is listed, and the dashed boxes indicate the locations of three initiator sequences (Inr). The closed boxes indicate the consensus sites for Sp1 binding, and consensus binding sites for E2F and v-myB are in boldface. (**B**) The indicated regions of 5′ flanking DNA were fused with firefly luciferase and assayed for promoter activity by measuring luminescence in extracts prepared from transfected CV1 cells. Expression was normalized to that of the *Renilla* luciferase gene. The constructions are numbered relative to the beginning of the longest mRNA that we identified. The substantial luciferase activity of −1762 to +1 identifies this region as a functional *SNX1* promoter. Deletion of bp −1162 to −159 resulted in only a 13.9% loss of basal promoter activity, whereas deletion of −159 to +1 resulted in a 98% loss of activity. The open boxes in the diagrams indicate the positions of the SP1/E2F boxes.

Chromosomal localization of SNX1

Fluorescence *in situ* hybridization with 6K1 and the two additional genomic clones was used to determine the chromosomal location of *SNX1* (data not shown). The hybridization efficiency was 85% to 91% and localized *SNX1* to human chromosome 15q22. Similarly, the International RH Mapping Consortium (Deloukas *et al.*, 1998; Schuler *et al.*, 1996) mapped *SNX1* to chromosome 15 in the reference interval D15S117–D15S159. We also identified the *SNX1* gene in an unordered working draft of sequences from human chromosome 15 submitted to GenBank by the Washington University Genome Sequencing Center (gi7024110). Using our 6K1 map as a guide, we ordered and oriented five of these contigs into a 99.2%-sequenced 70,473-bp map that contained the *SNX1* structural gene between bp 9860 and 53415. By comparison, our map based on 6K1 contained the *SNX1* structural gene between bp 1765 and 44624 and was 39.8% sequenced. The structural gene is 1.6% larger in the map derived from gi7024110 than in the map derived from 6K1. This difference is secondary to the insertion of runs of N in the GenBank entry to represent gaps of unknown size and to our estimation of fragment sizes in unsequenced 6K1 regions by agarose gel electrophoresis.

Using the map derived from gi7024110, BLAST searches against the human EST database were performed using overlapping 10,000-base segments to identify additional coding regions in the *SNX1* gene. Although there were several instances of ESTs within the *SNX1* coding regions, these contained repetitive sequences. Thus, there do not appear to be any additional genes within the *SNX1* structural gene. However, we did identify genes that flank the *SNX1* structural gene. Approximately 2.8 kb 5′ of *SNX1* intron 1 is a ~5-kb segment containing two exons and an intron represented by 13 ESTs (e.g., gi9124080) for a gene of unknown function. Approximately 6 kb 3′ of the end of exon 15 is a 400-bp segment containing two exons and an intron also represented by 13 ESTs (e.g., gi3308527), but these ESTs contain L1 repetitive elements. Even further 3′, approximately 13.5 kb downstream of the end of exon 15 are four exons and three introns from a gene of unknown function represented by a single EST (gi1183071) derived from fetal brain. This defines the relation of *SNX1* to adjacent genes on chromosome 15 and indicates that there are unlikely to be additional enhancer elements upstream of the *SNX1* gene sequences that we analyzed between −1761 and +1.

DISCUSSION

The complete human *SNX1* gene is localized to a 43-kb segment of human chromosome 15q22. Translocations and deletions involving this region of chromosome 15 are associated with acute myeloid leukemia, especially the PML translocation (Mitelman *et al.*, 1997). The *SNX1* gene is complex and is divided between at least 15 exons. The SNX1 mRNA is widely expressed in human tissues, and we demonstrated that the transcript is subject to alternative splicing that leads to exon skipping. The intron–exon boundaries we identified in the *SNX1* gene corresponded to those deduced from observed deletions of exons 3 or 4 or of exon 12 and establish that the SNX1 transcript is subject to alternative splicing. These two exon-skipping events were analyzed by PCR of cDNA arrays, and one event (exon 12 skipping) was found to be rare. The other (exon 3, 4 skipping) was frequently detected, but in no case was the exon-skipped species more abundant than the complete mRNA containing all 15 exons. Expression of SNX1 mRNA was lower in fetal brain and liver than in adult brain or liver. In the case of liver, there also appeared to be developmental regulation of exon 3, 4 skipping: the proportion of exon 3, 4-skipped mRNA was higher in fetal liver than in adult liver. A more definitive analysis of differential splicing by quantitative PCR of RNA extracted from liver at different stages of development will be needed to determine if this is biologically significant. In general, the intensity of PCR products derived from the 5′ and 3′ ends of the SNX1 mRNA were comparable. However, in several tissues, the intensity of the PCR product derived from the 3′ end of the SNX1 mRNA was higher than that for the 5′ end. This result may indicate alternative splicing of exon 1 against which one of the 5′-end PCR primers was complementary. However, no human ESTs were identified that reflected this splicing event.

A 1762-bp 5′ flanking DNA fragment had robust promoter activity when fused to luciferase. Promoter activity was completely abolished by the removal of the proximal 160 bp of 5′ flanking DNA. When fused with luciferase, this 160-bp region retained the robust activity of the 1762-bp 5′ flanking DNA fragment. Thus, the region −160 to +1 appears both necessary and sufficient to drive *SNX1* gene transcription. The region −160 to +1 was further subdivided into an Inr-containing domain and an SP1/E2F box. The deletion of either of these regions resulted in a substantial (>50%) loss of activity. Deletion of the Inr-containing domain may have activated a cryptic Inr sequence located just upstream of the SP1/E2F box. However, this Inr was inactive if the SP1/E2F boxes were also deleted.

Functional analysis of SNX1 has been limited to overexpression studies in cultured cell lines. The identification and characterization of the human *SNX1* gene will permit the construction of targeting vectors for gene disruption by homologous recombination (Mansour *et al.*, 1988) and will also facilitate the further analysis of potential *SNX1* promoter regulation by hormones and potential alternative splicing of the transcript.

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